STUDIES ON THE GLYCOGEN OF *M. TUBERCULOSIS* (HUMAN STRAIN)

by

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There have been frequent observations [LEVENE (1904); HEIDELBERGER AND MENZEL (1937)] regarding the presence of glycogen in numerous microbial cells and various preparations including the well-defined material of CHARGAFF AND MOORE (1944) from the tubercle bacillus have been described.

The present paper is a further contribution to the chemistry of "tubercle glycogen".

EXPERIMENTAL

EXTRACTION WITH ALKALI OF DEGRADED GLYCOGEN FROM *M. tuberculosis*

Moist cells (2000 g) killed by steaming for 3 hours, were extracted exhaustively with acetone in a continuous extractor. During this process, a white crystalline solid (2 g) accumulated, apparently by sublimation in the condenser of the apparatus even when CO_2 was completely excluded. The substance was identified chemically as ammonium carbonate and this was confirmed by X-ray powder photographs. The acetone-treated cells were then extracted exhaustively with ether. The defatted organisms (210 g) were extracted with a solution of sodium hydroxide (1 N, 2 l) for 24 hours at 90° C. The insoluble residue was removed and the liquor acidified with acetic acid. On addition of ethanol (4 vols) a precipitate of crude carbohydrate material was obtained. This was redissolved in a little dilute acetic acid ($\frac{N}{20}$) and dialysed for 48 hours. After centrifuging the polysaccharide (15 g) was reprecipitated with excess ethanol and was dried by washing with ethanol and ether. This product had $[\alpha]_D^{19}$ + 52° (in water), Ash 55%; it gave a strongly positive Dische and Molisch tests, and gave a reddish-brown coloration on addition of dilute iodine solution.

FRACTIONATION OF DEGRADED GLYCOGEN

The impure carbohydrate (10 g) was separated into fractions by dissolving the crude product in water (100 g) and adding ethanol dropwise to the stirred solution until some precipitation occurred. The fraction was separated and more ethanol cautiously added to the supernatant to give a further precipitation. In this way, a series of fractions were obtained as shown in Table I.

It was clear that the glycogen was contained almost wholly in fractions 2 and 3. These were combined with other iodine-staining fractions and refractionated by alcoholic precipitation as before (Table II). The final material, Fractions 11 and 12, gave a strong red coloration with iodine and were used for the investigation.

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TABLE I

FRACTIONATION OF CRUDE CARBOHYDRATE (10 g)

Fraction obtained by adding ether and ethanol	Weight of precipitated g	$[\alpha]_D^{19}$*	Bial test	Dische test	Iodine coloration	Polysaccharide
1	0.05	+20°	—	—	—	Mainly inorganic
2	0.23	+67°	—	±	brown	Impure degraded bacterial glycogen
3	0.42	+85°	+	±	reddish-brown	
4	2.8	+86°	++	+++		Specific polysaccharide (HAWORTH, KENT, AND STACEY, 1948a)
5	0.68	+72°	+++	+		
6	0.18	+58°	+++	+	—	Mixed Fractions
7	0.45	+40°	++	+	—	
8	4.0	+30°	++	+++	—	Specific Polysaccharide (HAWORTH, KENT, AND STACEY, 1948b)
9	1.51	+27°	++	++	—	
10	0.23	+35°	+	±	—	Not examined

* Corrected for ash.

TABLE II

FURTHER FRACTIONATION OF DEGRADED GLYCOGEN

Fraction No.	Amount of ethanol added (ml)	$[\alpha]_D^{20}$ in water	Weight g	Iodine coloration	Dische test	Polysaccharide
11	6	+163°	0.25	++	—	Degraded bacterial glycogen
12	10.2	+171°	0.46	++	—	
13	13.0	+100°	0.21	±	—	Mixture
14	15.2	+ 89°	0.08	—	—	Somatic polysaccharide
15	4	+ 78°	0.09	—	±	Somatic polysaccharide
16	25	+ 52°	0.10	—	±	Somatic polysaccharide

Material corresponding to Fractions 11 and 12 was collected and purified further by repeated fractional precipitation from solvents, dialysis, etc., until an essentially hydrogenous, electrophoretically immobile product, $[\alpha]_D^{20°} + 178°$ was obtained. It gave a typical "glycogen" stain with iodine.

HYDROLYSIS WITH DILUTE MINERAL ACID

The polysaccharide (15 mg) dried for 4 hours at 60°, was dissolved in sulphuric acid (0.5 ml of 0.5 N), and heated at 100° C. The hydrolysis was complete in 4 hours ($[\alpha]_D^{18°} + 178° \rightarrow + 58°$ equilibrium value).

PAPER CHROMATOGRAPHY OF THE HYDROLYSED POLYSACCHARIDE

The hydrolysate from 15 mg of glycogen was made neutral with barium carbonate, filtered and concentrated to dryness. The residue was taken up in two drops of water and a specimen of this solution was transferred (by a capillary tube) to a strip of Whatman No. 1 filter paper (10 cm·30 cm). Alongside was placed a similar amount of a 2% solution of glucose in water. The chromatography was carried out over 12 hours at room temperature using butanol (40%)/ethanol(10%)/water(50%).

The chromatogram, after development with dilute ammoniacal silver nitrate solution showed clearly the presence of glucose only.

ISOLATION OF GLUCOSE ANILIDE

The remaining neutralized hydrolysate (see above) was dried and refluxed for 2 hours with dry ethanol (2 ml) containing 10 mg of aniline. After filtration, a solid separated which after recrystallisation had m.p. 145–6° alone or in admixture with authentic specimen of D-glucose anilide.

REDUCING SUGAR CONTENT

The polysaccharide (2.0 mg) was hydrolysed by $\frac{N}{2}$ H_2SO_4 (1 ml) at 100° C for 4 hours, neutralized (p_H 8) with sodium carbonate and the amount of reducing sugar estimated by titration with Schaffer-Hartmann reagents, giving 2·17 mg of glucose, *i.e.*, 98% reducing sugar.

1. *Attempted Amylolytic degradation of the degraded glycogen*

The dried glycogen (2.5 mg) dissolved in 0.5 ml of phosphate buffer at p_H 7 was treated with 0.5 ml of salivary amylase at 25° C. After 35 minutes the iodine coloration had completely disappeared and the resulting solution was reducing to Fehling's solution. Identical results were obtained with β-amylase from soya beans.

2. *"Starter" function* (SITCH AND PEAT-private communication)

The polysaccharide (5 mg) was dissolved in 1 ml of citrate buffer at p_H 6, containing 1 ml of 0.1 M potassium glucose-1-phosphate and this solution was treated with 1 ml of a purified potato phosphorylase (P-enzyme) for 12 minutes at 35° C. After removal

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of the protein with trichloroacetic acid (5 ml 6%) the inorganic phosphorus liberated was estimated colorimetrically.

Phosphorus liberated = 7.4 (mg/100 ml)

$$\text{Activating power ratio} = 0.22 = \frac{\text{mg/ml phosphorus liberated by 5 mg polysaccharide}}{\text{mg/ml phosphorus in control}}$$

ISOLATION OF GLYCOGEN BY MEANS OF TRICHLOROACETIC ACID

Moist organisms (200 g) were ground with trichloroacetic acid (0.1 N; 200 ml) for 17 hours at room temperature. The cell debris was removed by centrifuging and by passing the resulting liquid through a Seitz filter. The filtrate was dialysed in cellophane against running water for 64 hours. After being centrifuged, the solution was then concentrated by evaporation to 10 ml. This solution was dialysed for a further 24 hours and finally it was freeze-dried.

The product (80 mg) gave negative tests for protein and nucleic acid, but imparted the characteristic coloration with iodine and gave a strongly positive Molisch test. The optical activity could not be determined initially due to the opalescence of the solution.

% N = 0.6 after precipitation of the polysaccharide from aqueous solution by addition of alcohol (3 vols).

The substance underwent hydrolysis readily with dilute mineral acid $\frac{N}{2}$ H_2SO_4 [α_D^{20} + 92° (after 10 minutes), + 57° (after $5^3/_4$ hours) (c = 0.5). The resulting solution, after neutralization was concentrated and examined chromatographically on filter paper. As before, only one component, glucose appeared and this had R_F, 0.187.

The fraction was examined by the method described for any possible "starter" activity. Under the conditions of the experiment, there was no detectable liberation of phosphorus.

POTASSIUM PERIODATE OXIDATIONS

a) On degraded glycogen

37.35 mg of the fractionated polysaccharide were dissolved in 50 ml of 10% potassium chloride to which was added 5 ml of sodium metaperiodate (0.494 M). The mixture was shaken and allowed to stand at room temperature.

At intervals of 24 hours, 5 ml portions were withdrawn, diluted with a little distilled water and 0.1 ml of ethylene glycol added. The formic acid liberated was titrated with 0.006 M sodium hydroxide using methyl red as the indicator.

The liberation of acid appeared to be complete after 140 hours.

100 mg glycogen = 1.72 ml 0.006 N NaOH, thus

3 mols formic acid = 1870 g polysaccharide

= 11.6 glucose units

b) On NaOH extracted glycogen (unfractionated material)

0.03735 g = 0.64 ml · 11 ml 0.0065 N NaOH

$$\text{Therefore 3 mols formic acid} = \frac{0.03735 \cdot 3 \cdot 1000}{0.64 \cdot 11 \cdot 0.0065 \cdot 162} = \underline{17.9} \text{ glucose units}$$

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TRICHLOROACETIC ACID EXTRACTED GLYCOGEN

0.02789 g dissolved in 50 ml 10% KCl + 5 ml 0.4 M $NaIO_4$
After 140 hours

$$0.02786 \text{ g} = 0.48 \text{ ml} \cdot 11 \text{ ml } 0.0065 \text{ N NaOH}$$

$$\text{Therefore 3 mols formic acid} = \frac{0.02786 \cdot 3 \cdot 1000}{0.48 \cdot 11 \cdot 0.0065 \cdot 162} = \underline{15.1} \text{ glucose units}$$

DISCUSSION

The presence of a polysaccharide, which in solution gave a coloration with iodine similar to that given by glycogen, was described by LEVENE (1904) who obtained it by extraction of *M. tuberculosis* cells with dilute sodium chloride solution. Further mention of such a carbohydrate was made by WARKANY (1925) and a method was devised by LAIDLAW AND DUDLEY (1925) for separating it from the pentose-containing specific polysaccharides of the organism. These workers showed that on acid hydrolysis of the polysaccharide glucose only was produced.

Later workers (CHARGAFF AND SCHAEFER, 1935; HEIDELBERGER AND MENZEL, 1937) have reported the presence of this "glycogen" and have employed salivary enzymes for removing it from the other carbohydrates of the organism. Further references to this polysaccharide are given in a recent review by KENT AND STACEY (1948).

A detailed examination of the "glycogen" of the avian strain of *M. tuberculosis* has been carried out by CHARGAFF AND MOORE (1944). Their product was isolated from the defatted organism by use of a borate buffer and also by the well established method of extraction of the cells with a dilute solution of trichloroacetic acid. The glycogen which gave readily the characteristic coloration with iodine, appeared to be in a relatively undegraded state since an examination by the electrophoretic and ultracentrifuge methods showed that it had a very high particle weight, and an axial ration of 11. The authors showed furthermore that the glycogen after mild acid hydrolysis gave a reducing value of 102% calculated as glucose. The evidence suggested that the carbohydrate was indeed a true glycogen having a complex structure. No chemical evidence was provided concerning the length of the chains or their degree of branching.

CHARGAFF (1947) has reported further that this glycogen fraction was degraded by β-amylase but did not itself behave as a "starter" in the synthesis of glycogen from glucose-1-phosphate by the action of muscle phosphorylase (CORI, 1939), although after degradation by dilute alkali, the glycogen was able to function as a starter. It is known that only small chains of α-1:4-linked polyglucosans function as starters either with muscle phosphorylase or with potato phosphorylase (BOURNE, PEAT, AND SITCH, private communication).

No immunological function has yet been ascribed to any glycogen fraction from any strain of *M. tuberculosis*.

In the course of investigation of the carbohydrate constituents of the cells of *M. tuberculosis* (human strain) (HAWORTH, KENT, AND STACEY, 1948) it was found that a carbohydrate giving the characteristic red staining reaction with iodine, was obtained by extraction of defatted cells with dilute alkali. The substance was separated from the contaminating specific polysaccharides and nucleic acids by fractional precipitation by alcohol from an aqueous solution. The purified carbohydrate was considered to be a true

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glycogen because of the following properties: a) in solution, it possessed the characteristic red staining property of a glycogen, b) it gave typically opalescent aqueous solutions and had $[\alpha]_D^{20°}$ + 178° in water, c) hydrolysis proceeded rapidly with dilute acid $[\alpha]_D^{20°}$ + 178° → $[\alpha]_D^{20°}$ + 58° forming a reducing syrup, in which glucose was identified as the sole constituent, d) the substance was completely degraded by β-amylase to a reducing sugar, e) it behaved as a starter in a phosphorylase synthesis.

The stages in the purification of the glycogen were followed polarimetrically and by the chromatographic examination on paper of hydrolysed test fractions of the substance (PARTRIDGE, 1946, 1947). By the latter method, the hydrolysate of the final purified glycogen showed only one constituent, having R_F, 0.186 in a butanol-ethanol-water system (R_F 0.18 for glucose under the same conditions). Estimation of the total reducing sugar by the SCHAFFER-HARTMANN method in this hydrolysate gave a value 98% calculated as glucose.

The presence of glucose was confirmed by the isolation of crystalline glucose anilide from the hydrolysed polysaccharide.

The optical rotation and rate of hydrolysis of the final glycogen fraction were in close accord with the corresponding data given by glycogen fractions from other sources.

Enzymatic degradation proceeded rapidly at 37° C using β-amylase from saliva or Soya beans. The starter function was determined using potato phosphorylase and potassium glucose-1-phosphate at 35° C. Controls carried out simultaneously were negative in all cases.

Attempts to demonstrate the presence of a phosphorylase in the culture filtrate of the human strain organism at various stages of growth were unsuccessful.

Oxidation of the glycogen at room temperature with potassium periodate using HIRST's (1945) extension of BARRY's (1942) method was of some interest. By this technique, each glycogen chain (assuming that there is no branching of the chain) gives rise to three moles of formic acid. Thus, the chain length of the glycogen under consideration appeared to consist of 12-glucopyranose units. Satisfactory controls were carried out with glycogen fractions isolated from guinea pig liver and rabbit liver.

For comparison, a further glycogen fraction has been isolated from the human strain organism using a method analogous to that of CHARGAFF AND MOORE (*loc. cit.*) namely by the extraction of defatted organisms with dilute trichloracetic acid. By this means a substance was obtained, which after reprecipitation by alcohol from aqueous solution was nitrogen free, and had the characteristic staining property with iodine. In aqueous solution, the high degree of opalescence prevented the satisfactory measurement of its optical rotation. After hydrolysis with dilute mineral acid, the resulting solution $[\alpha]_D^{20}$ + 60° was strongly reducing to Fehling's solution, and showed only a single component reducing sugar when examined by paper chromatography. The initial fraction in this case did not behave as a starter in phosphorylase synthesis.

Oxidation of this fraction with potassium periodate gave results similar to those given by the alkali-extracted material though it differed in having a chain-length of 15 glucose units. It would appear that the glycogen isolated by the trichloroacetic acid method is a more complex macromolecule than the alkali-extracted material.

SUMMARY

A description is given of the isolation of a degraded glycogen from the mixed polysaccharide of *M. tuberculosis* human strain and it would appear to possess the usual chain units of 10–12 glucose residues.

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RÉSUMÉ

On décrit l'isolation d'un glycogène dégradé à partir du mélange de polysaccharides de souche humaine de *M. tuberculosis*, lequel paraît posséder les chaînes habituelles de 10–12 résidus de glucose.

ZUSAMMENFASSUNG

Die Isolierung eines degradierten Glykogens aus dem gemischten Polysaccharid von menschlichem *M. tuberculosis* wird beschrieben. Es scheint die übliche Ketteneinheit von 10–12 Glucoseresten zu enthalten.

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